

ENTEROHEMORRHAGIC *ESCHERICHIA COLI* VACCINE

Cross-Reference to Related Application

This application claims the benefit under 35 USC §119(e)(1) of provisional patent application serial no. 60/259,818, filed January 4, 2001, which application is incorporated herein by reference in its entirety.

Field of the Invention

The present invention relates to compositions and methods for eliciting an immune response in mammals against enterohemorrhagic *Escherichia coli*. In particular, the invention relates to the use of cell culture supernatants for treating and preventing enterohemorrhagic *E. coli* colonization of mammals.

Background of The Invention

Enterohemorrhagic *Escherichia coli* (EHEC), also called Shiga toxin *E. coli* (STEC) and verotoxigenic *E. coli* (VTEC) are pathogenic bacteria that cause diarrhea, hemorrhagic colitis, hemolytic uremic syndrome, kidney failure and death in humans. While many Shiga-like toxin-producing EHEC strains are capable of causing disease in humans, those of serotype O157:H7 cause the majority of human illness. This organism is able to colonize the large intestine of humans by a unique mechanism in which a number of virulence determinants are delivered to host cells via a type III secretion system, including the translocated Intimin receptor, Tir (DeVinney et al., *Infect. Immun.* (1999) 67:2389). In particular, these pathogens secrete virulence determinants EspA, EspB and EspD that enable delivery of Tir into intestinal cell membranes. Tir is integrated into the host cell membrane where it serves as the receptor for a bacterial outer membrane protein, Intimin. Tir-Intimin binding attaches EHEC to the intestinal cell surface and triggers actin cytoskeletal rearrangements beneath adherent EHEC that results in pedestal formation. EspA, EspB, Tir and

Intimin are each essential for the successful colonization of the intestine by EHEC.

Although EHEC colonize the intestine of ruminants and other mammals, they generally do not cause overt disease in these animals. However, contamination of meat and water by the EHEC serotype O157:H7 (hereinafter, "EHEC O157:H7") is responsible for about 50,000 cases of EHEC O157:H7 infection in humans annually in the United States and Canada that result in approximately 500 deaths. In 1994, the economic cost associated with EHEC O157:H7 infection in humans was estimated to be over 5 billion dollars annually.

The first documented EHEC O157:H7 outbreak traced to contaminated meat occurred in 1982. Subsequently, it was demonstrated that healthy ruminants including, but not limited to, cattle, dairy cows and sheep, could be infected with EHEC O157:H7. In fact, USDA reports indicate that up to 50% of cattle are carriers of EHEC O157:H7 at some time during their lifetime and, therefore, shed EHEC O157:H7 in their feces.

Because of the bulk processing of slaughtered cattle and the low number of EHEC O157:H7 (10-100) necessary to infect a human, EHEC O157:H7 colonization of healthy cattle remains a serious health problem. To address this problem, research has focused on improved methods for detecting and subsequently killing EHEC O157:H7 at slaughter, altering the diet of cattle to reduce the number of intestinal EHEC O157:H7 and immunizing animals to prevent EHEC O157:H7 colonization (Zacek D. Animal Health and Veterinary Vaccines, Alberta Research Counsel, Edmonton, Canada, 1997). Recently, the recombinant production and use of EHEC O157:H7 proteins including recombinant EspA (International Publication No. WO 97/40063), recombinant TIR (International Publication No. WO 99/24576), recombinant EspB and recombinant Intimin (Li et al., *Infec. Immun.* (2000) 68:5090-5095) have been described. However, production and purification of recombinant proteins in amounts sufficient for use as antigens is both difficult and expensive. At the present time, there is no effective method for blocking EHEC O157:H7 colonization of cattle and other mammals and, thereby, for reducing shedding of EHEC into the environment.

Therefore, there is a need for new compositions and methods for treating and preventing EHEC disease, as well as for reducing EHEC colonization of mammals in order to reduce the

incidence of health problems associated with EHEC-contaminated meat and water.

Summary of The Invention

5 The present invention satisfies the above need by providing such compositions and methods. In particular, the methods of the present invention make use of a composition comprising a cell culture supernatant (hereinafter "CCS") derived from an EHEC culture to elicit an immune response against one or more EHEC secreted antigens, thereby treating and/or preventing EHEC infection and/or reducing EHEC colonization of the mammal. The compositions can be delivered with or without a coadministered adjuvant. In certain embodiments, EspA and Tir comprise at least 10 20% of the cell culture supernatant protein. The EHEC culture supernatant may be derived from any EHEC serotype, but is preferably obtained from a culture of EHEC O157:H7 and/or EHEC O157:NM (non-motile). The cell culture supernatant of the present invention is easy and relatively inexpensive to prepare and is effective at dose regimens that have minimal toxicity.

15 EspA, EspB, Tir and Intimin are necessary for activation (A) of host epithelial cell signal transduction pathways and for the intimate attachment (E) of EHEC to host epithelial cells. Therefore, without being bound by the following hypothesis, it is thought that administration of the CCS of the present invention to a mammal stimulates an immune response against one or more secreted antigens, such as EspA and Tir, that blocks attachment of the EHEC to intestinal epithelial cells.

20 Accordingly, it is an object of the present invention to provide a vaccine effective to stimulate an immune response against EHEC secreted antigens, thereby treating and/or preventing EHEC disease in a mammal.

Another object is to provide a vaccine effective to reduce, prevent and/or eliminate EHEC colonization of a ruminant or other mammal.

25 Another object is to reduce the number of animals shedding EHEC into the environment.

Another object is to reduce the number of EHEC shed into the environment by an infected animal.

Another object is reduce the time during which EHEC are shed into the environment by an

infected animal.

Another object is reduce EHEC contamination of the environment.

Another object is reduce EHEC contamination of meat and/or water.

Another object is to treat, prevent and/or reduce EHEC infections in humans.

5 Another object is to provide a vaccine effective as an adjunct to other biological anti-EHEC agents.

Another object is to provide a vaccine effective as an adjunct to chemical anti-EHEC agents.

Another object is to provide a vaccine effective as an adjunct to biologically engineered anti-EHEC agents.

10 Another object is to provide a vaccine effective as an adjunct to nucleic acid-based anti-EHEC agents.

Another object is to provide a vaccine effective as an adjunct to recombinant protein anti-EHEC agents.

15 Another object is to provide a vaccination schedule effective to reduce EHEC colonization of a ruminant.

Another object is to provide a vaccination schedule effective to reduce EHEC shedding by a ruminant.

Another object is to provide a vaccine effective to reduce EHEC O157 colonization of cattle, such as colonization of EHEC O157:H7 and/or EHEC O157:NM.

20 Another object is to provide a vaccine effective to prevent EHEC O157 colonization of cattle, such as colonization of EHEC O157:H7 and/or EHEC O157:NM.

Another object is to provide a vaccine effective to eliminate EHEC O157 colonization of cattle, such as colonization of EHEC O157:H7 and/or EHEC O157:NM.

25 Another object is to reduce the number of cattle shedding EHEC O157 into the environment, such as shedding of EHEC O157:H7 and/or EHEC O157:NM.

Another object is to reduce the number of EHEC O157 shed into the environment by infected cattle, such as shedding of EHEC O157:H7 and/or EHEC O157:NM.

Another object is reduce the time during which EHEC O157 are shed into the environment

by infected cattle, such as shedding of EHEC O157:H7 and/or EHEC O157:NM.

Another object is to provide a vaccine effective as an adjunct to other anti-EHEC O157 agents.

Another object is to provide a vaccination schedule effective to reduce EHEC O157
5 colonization of cattle.

Another object is to provide a vaccination schedule effective to reduce EHEC O157 shedding by cattle.

Thus, in one embodiment, the invention is directed to a vaccine composition comprising an enterohemorrhagic *Escherichia coli* (EHEC) cell culture supernatant and an immunological
10 adjuvant. In certain embodiments, the EHEC is EHEC O157:H7 and/or EHEC O157:NM. In additional embodiments, the immunological adjuvant comprises an oil-in-water emulsion, such as a mineral oil and dimethyldioctadecylammonium bromide. In yet additional embodiments, the immunological adjuvant is VSA3. The VSA3 may be present at a concentration of about 20% to about 40% (v/v), such as at a concentration of 30% (v/v).

15 In still further embodiments, the vaccine composition further comprises one or more recombinant or purified EHEC secreted antigens selected from the group consisting of EspA, EspB, EspD and Tir. In other embodiments, EspA + Tir comprise at least 20% of the cell protein present in the composition.

20 In further embodiments, the subject invention is directed to methods for eliciting an immunological response in a mammal against a secreted enterohemorrhagic *Escherichia coli* (EHEC) antigen. The method comprises administering to the mammal a therapeutically effective amount of a composition comprising an EHEC cell culture supernatant. In certain embodiments, the EHEC is EHEC O157:H7 and/or EHEC O157:NM. In additional embodiments, the mammal is a human or a ruminant, such as a bovine subject. In yet further embodiments, the composition
25 further comprises an immunological adjuvant, such as an oil-in-water emulsion which comprises e.g., a mineral oil and dimethyldioctadecylammonium bromide. In additional embodiments, the adjuvant is VSA3. The compositions may further comprise one or more recombinant or purified EHEC secreted antigens selected from the group consisting of EspA, EspB, EspD and Tir. In other

embodiments, EspA + Tir comprise at least 20% of the cell protein present in the composition.

In another embodiment, the invention is directed to a method for eliciting an immunological response in a ruminant against a secreted enterohemorrhagic *Escherichia coli* O157:H7 (EHEC O157:H7) antigen. The method comprises administering to the ruminant a therapeutically effective amount of a composition comprising an EHEC O157:H7 cell culture supernatant and VSA3. In additional embodiments, VSA3 is present in the composition at a concentration of about 20% to about 40% (v/v), such as at about 30% (v/v).

In still a further embodiment, the invention is directed to a method for reducing colonization of enterohemorrhagic *Escherichia coli* (EHEC) in a ruminant comprising administering to the ruminant a therapeutically effective amount of a composition comprising an EHEC cell culture supernatant and an immunological adjuvant.

In yet another embodiment, the invention is directed to a method for reducing shedding of enterohemorrhagic *Escherichia coli* (EHEC) from a ruminant comprising administering to the ruminant a therapeutically effective amount of a composition comprising an EHEC cell culture supernatant and an immunological adjuvant.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings. In addition, various references are set forth herein which describe in more detail certain procedures or compositions, and are therefore incorporated by reference in their entireties.

Brief Description of The Drawings

Figure 1 shows the electrophoretic profile of CCS proteins separated by polyacrylamide gel electrophoresis.

Figure 2 shows the electrophoretic profile of recombinant EspA, Tir, EspB and Intimin separated by polyacrylamide gel electrophoresis.

Figure 3 shows fecal shedding of EHEC O157:H7 by cattle immunized with a CCS vaccine following EHEC O157:H7 challenge.

Figure 4 depicts reactivation of fecal shedding of EHEC O157:H7 in previously infected

cattle.

Figure 5 shows the serological response to immunization with recombinant EspA + Tir vaccine and with recombinant EspB + Intimin vaccine.

Figure 6 depicts fecal shedding of EHEC O157:H7 following immunization with
5 recombinant EspA + Tir vaccine and with saline vaccine.

Figure 7 shows the number of animals shedding *E. coli* O157:H7 on each day of the vaccine trial described in Example 6. Bacteria were detected by direct plating of fecal samples which had been resuspended in saline on Sorbitol MaConkey agar supplemented with cefixime and tellurite. Solid bars, placebo group; hatched bars, EHEC vaccine group.

10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
90
95
100
105
110
115
120
125
130
135
140
145
150
155
160
165
170
175
180
185
190
195
200
205
210
215
220
225
230
235
240
245
250
255
260
265
270
275
280
285
290
295
300
305
310
315
320
325
330
335
340
345
350
355
360
365
370
375
380
385
390
395
400
405
410
415
420
425
430
435
440
445
450
455
460
465
470
475
480
485
490
495
500
505
510
515
520
525
530
535
540
545
550
555
560
565
570
575
580
585
590
595
600
605
610
615
620
625
630
635
640
645
650
655
660
665
670
675
680
685
690
695
700
705
710
715
720
725
730
735
740
745
750
755
760
765
770
775
780
785
790
795
800
805
810
815
820
825
830
835
840
845
850
855
860
865
870
875
880
885
890
895
900
905
910
915
920
925
930
935
940
945
950
955
960
965
970
975
980
985
990
995
1000
1005
1010
1015
1020
1025
1030
1035
1040
1045
1050
1055
1060
1065
1070
1075
1080
1085
1090
1095
1100
1105
1110
1115
1120
1125
1130
1135
1140
1145
1150
1155
1160
1165
1170
1175
1180
1185
1190
1195
1200
1205
1210
1215
1220
1225
1230
1235
1240
1245
1250
1255
1260
1265
1270
1275
1280
1285
1290
1295
1300
1305
1310
1315
1320
1325
1330
1335
1340
1345
1350
1355
1360
1365
1370
1375
1380
1385
1390
1395
1400
1405
1410
1415
1420
1425
1430
1435
1440
1445
1450
1455
1460
1465
1470
1475
1480
1485
1490
1495
1500
1505
1510
1515
1520
1525
1530
1535
1540
1545
1550
1555
1560
1565
1570
1575
1580
1585
1590
1595
1600
1605
1610
1615
1620
1625
1630
1635
1640
1645
1650
1655
1660
1665
1670
1675
1680
1685
1690
1695
1700
1705
1710
1715
1720
1725
1730
1735
1740
1745
1750
1755
1760
1765
1770
1775
1780
1785
1790
1795
1800
1805
1810
1815
1820
1825
1830
1835
1840
1845
1850
1855
1860
1865
1870
1875
1880
1885
1890
1895
1900
1905
1910
1915
1920
1925
1930
1935
1940
1945
1950
1955
1960
1965
1970
1975
1980
1985
1990
1995
2000
2005
2010
2015
2020
2025
2030
2035
2040
2045
2050
2055
2060
2065
2070
2075
2080
2085
2090
2095
2100
2105
2110
2115
2120
2125
2130
2135
2140
2145
2150
2155
2160
2165
2170
2175
2180
2185
2190
2195
2200
2205
2210
2215
2220
2225
2230
2235
2240
2245
2250
2255
2260
2265
2270
2275
2280
2285
2290
2295
2300
2305
2310
2315
2320
2325
2330
2335
2340
2345
2350
2355
2360
2365
2370
2375
2380
2385
2390
2395
2400
2405
2410
2415
2420
2425
2430
2435
2440
2445
2450
2455
2460
2465
2470
2475
2480
2485
2490
2495
2500
2505
2510
2515
2520
2525
2530
2535
2540
2545
2550
2555
2560
2565
2570
2575
2580
2585
2590
2595
2600
2605
2610
2615
2620
2625
2630
2635
2640
2645
2650
2655
2660
2665
2670
2675
2680
2685
2690
2695
2700
2705
2710
2715
2720
2725
2730
2735
2740
2745
2750
2755
2760
2765
2770
2775
2780
2785
2790
2795
2800
2805
2810
2815
2820
2825
2830
2835
2840
2845
2850
2855
2860
2865
2870
2875
2880
2885
2890
2895
2900
2905
2910
2915
2920
2925
2930
2935
2940
2945
2950
2955
2960
2965
2970
2975
2980
2985
2990
2995
3000
3005
3010
3015
3020
3025
3030
3035
3040
3045
3050
3055
3060
3065
3070
3075
3080
3085
3090
3095
3100
3105
3110
3115
3120
3125
3130
3135
3140
3145
3150
3155
3160
3165
3170
3175
3180
3185
3190
3195
3200
3205
3210
3215
3220
3225
3230
3235
3240
3245
3250
3255
3260
3265
3270
3275
3280
3285
3290
3295
3300
3305
3310
3315
3320
3325
3330
3335
3340
3345
3350
3355
3360
3365
3370
3375
3380
3385
3390
3395
3400
3405
3410
3415
3420
3425
3430
3435
3440
3445
3450
3455
3460
3465
3470
3475
3480
3485
3490
3495
3500
3505
3510
3515
3520
3525
3530
3535
3540
3545
3550
3555
3560
3565
3570
3575
3580
3585
3590
3595
3600
3605
3610
3615
3620
3625
3630
3635
3640
3645
3650
3655
3660
3665
3670
3675
3680
3685
3690
3695
3700
3705
3710
3715
3720
3725
3730
3735
3740
3745
3750
3755
3760
3765
3770
3775
3780
3785
3790
3795
3800
3805
3810
3815
3820
3825
3830
3835
3840
3845
3850
3855
3860
3865
3870
3875
3880
3885
3890
3895
3900
3905
3910
3915
3920
3925
3930
3935
3940
3945
3950
3955
3960
3965
3970
3975
3980
3985
3990
3995
4000
4005
4010
4015
4020
4025
4030
4035
4040
4045
4050
4055
4060
4065
4070
4075
4080
4085
4090
4095
4100
4105
4110
4115
4120
4125
4130
4135
4140
4145
4150
4155
4160
4165
4170
4175
4180
4185
4190
4195
4200
4205
4210
4215
4220
4225
4230
4235
4240
4245
4250
4255
4260
4265
4270
4275
4280
4285
4290
4295
4300
4305
4310
4315
4320
4325
4330
4335
4340
4345
4350
4355
4360
4365
4370
4375
4380
4385
4390
4395
4400
4405
4410
4415
4420
4425
4430
4435
4440
4445
4450
4455
4460
4465
4470
4475
4480
4485
4490
4495
4500
4505
4510
4515
4520
4525
4530
4535
4540
4545
4550
4555
4560
4565
4570
4575
4580
4585
4590
4595
4600
4605
4610
4615
4620
4625
4630
4635
4640
4645
4650
4655
4660
4665
4670
4675
4680
4685
4690
4695
4700
4705
4710
4715
4720
4725
4730
4735
4740
4745
4750
4755
4760
4765
4770
4775
4780
4785
4790
4795
4800
4805
4810
4815
4820
4825
4830
4835
4840
4845
4850
4855
4860
4865
4870
4875
4880
4885
4890
4895
4900
4905
4910
4915
4920
4925
4930
4935
4940
4945
4950
4955
4960
4965
4970
4975
4980
4985
4990
4995
5000
5005
5010
5015
5020
5025
5030
5035
5040
5045
5050
5055
5060
5065
5070
5075
5080
5085
5090
5095
5100
5105
5110
5115
5120
5125
5130
5135
5140
5145
5150
5155
5160
5165
5170
5175
5180
5185
5190
5195
5200
5205
5210
5215
5220
5225
5230
5235
5240
5245
5250
5255
5260
5265
5270
5275
5280
5285
5290
5295
5300
5305
5310
5315
5320
5325
5330
5335
5340
5345
5350
5355
5360
5365
5370
5375
5380
5385
5390
5395
5400
5405
5410
5415
5420
5425
5430
5435
5440
5445
5450
5455
5460
5465
5470
5475
5480
5485
5490
5495
5500
5505
5510
5515
5520
5525
5530
5535
5540
5545
5550
5555
5560
5565
5570
5575
5580
5585
5590
5595
5600
5605
5610
5615
5620
5625
5630
5635
5640
5645
5650
5655
5660
5665
5670
5675
5680
5685
5690
5695
5700
5705
5710
5715
5720
5725
5730
5735
5740
5745
5750
5755
5760
5765
5770
5775
5780
5785
5790
5795
5800
5805
5810
5815
5820
5825
5830
5835
5840
5845
5850
5855
5860
5865
5870
5875
5880
5885
5890
5895
5900
5905
5910
5915
5920
5925
5930
5935
5940
5945
5950
5955
5960
5965
5970
5975
5980
5985
5990
5995
6000
6005
6010
6015
6020
6025
6030
6035
6040
6045
6050
6055
6060
6065
6070
6075
6080
6085
6090
6095
6100
6105
6110
6115
6120
6125
6130
6135
6140
6145
6150
6155
6160
6165
6170
6175
6180
6185
6190
6195
6200
6205
6210
6215
6220
6225
6230
6235
6240
6245
6250
6255
6260
6265
6270
6275
6280
6285
6290
6295
6300
6305
6310
6315
6320
6325
6330
6335
6340
6345
6350
6355
6360
6365
6370
6375
6380
6385
6390
6395
6400
6405
6410
6415
6420
6425
6430
6435
6440
6445
6450
6455
6460
6465
6470
6475
6480
6485
6490
6495
6500
6505
6510
6515
6520
6525
6530
6535
6540
6545
6550
6555
6560
6565
6570
6575
6580
6585
6590
6595
6600
6605
6610
6615
6620
6625
6630
6635
6640
6645
6650
6655
6660
6665
6670
6675
6680
6685
6690
6695
6700
6705
6710
6715
6720
6725
6730
6735
6740
6745
6750
6755
6760
6765
6770
6775
6780
6785
6790
6795
6800
6805
6810
6815
6820
6825
6830
6835
6840
6845
6850
6855
6860
6865
6870
6875
6880
6885
6890
6895
6900
6905
6910
6915
6920
6925
6930
6935
6940
6945
6950
6955
6960
6965
6970
6975
6980
6985
6990
6995
7000
7005
7010
7015
7020
7025
7030
7035
7040
7045
7050
7055
7060
7065
7070
7075
7080
7085
7090
7095
7100
7105
7110
7115
7120
7125
7130
7135
7140
7145
7150
7155
7160
7165
7170
7175
7180
7185
7190
7195
7200
7205
7210
7215
7220
7225
7230
7235
7240
7245
7250
7255
7260
7265
7270
7275
7280
7285
7290
7295
7300
7305
7310
7315
7320
7325
7330
7335
7340
7345
7350
7355
7360
7365
7370
7375
7380
7385
7390
7395
7400
7405
7410
7415
7420
7425
7430
7435
7440
7445
7450
7455
7460
7465
7470
7475
7480
7485
7490
7495
7500
7505
7510
7515
7520
7525
7530
7535
7540
7545
7550
7555
7560
7565
7570
7575
7580
7585
7590
7595
7600
7605
7610
7615
7620
7625
7630
7635
7640
7645
7650
7655
7660
7665
7670
7675
7680
7685
7690
7695
7700
7705
7710
7715
7720
7725
7730
7735
7740
7745
7750
7755
7760
7765
7770
7775
7780
7785
7790
7795
7800
7805
7810
7815
7820
7825
7830
7835
7840
7845
7850
7855
7860
7865
7870
7875
7880
7885
7890
7895
7900
7905
7910
7915
7920
7925
7930
7935
7940
7945
7950
7955
7960
7965
7970
7975
7980
7985
7990
7995
8000
8005
8010
8015
8020
8025
8030
8035
8040
8045
8050
8055
8060
8065
8070
8075
8080
8085
8090
8095
8100
8105
8110
8115
8120
8125
8130
8135
8140
8145
8150
8155
8160
8165
8170
8175
8180
8185
8190
8195
8200
8205
8210
8215
8220
8225
8230
8235
8240
8245
8250
8255
8260
8265
8270
8275
8280
8285
8290
8295
8300
8305
8310
8315
8320
8325
8330
8335
8340
8345
8350
8355
8360
8365
8370
8375
8380
8385
8390
8395
8400
8405
8410
8415
8420
8425
8430
8435
8440
8445
8450
8455
8460
8465
8470
8475
8480
8485
8490
8495
8500
8505
8510
8515
8520
8525
8530
8535
8540
8545
8550
8555
8560
8565
8570
8575
8580
8585
8590
8595
8600
8605
8610
8615
8620
8625
8630
8635
8640
8645
8650
8655
8660
8665
8670
8675
8680
8685
8690
8695
8700
8705
8710
8715
8720
8725
8730
8735
8740
8745
8750
8755
8760
8765
8770
8775
8780
8785
8790
8795
8800
8805
8810
8815
8820
8825
8830
8835
8840
8845
8850
8855
8860
8865
8870
8875
8880
8885
8890
8895
8900
8905
8910
8915
8920
8925
8930
8935
8940
8945
8950
8955
8960
8965
8970
8975
8980
8985
8990
8995
9000
9005
9010
9015
9020
9025
9030
9035
9040
9045
9050
9055
9060
9065
9070
9075
9080
9085
9090
9095
9100
9105
9110
9115
9120
9125
9130
9135
9140
9145
9150
9155
9160
9165
9170
9175
9180
9185
9190
9195
9200
9205
9210
9215
9220
9225
9230
9235
9240
9245
9250
9255
9260
9265
9270
9275
9280
9285
9290
9295
9300
9305
9310
9315
9320
9325
9330
9335
9340
9345
9350
9355
9360
9365
9370
9375
9380
9385
9390
9395
9400
9405
9410
9415
9420
9425
9430
9435
9440
9445
9450
9455
9460
9465
9470
9475
9480
9485
9490
9495
9500
9505
9510
9515
9520
9525
9530
9535
9540
9545
9550
9555
9560
9565
9570
9575
9580
9585
9590
9595
9600
9605
9610
9615
9620
9625
9630
9635
9640
9645
9650
9655
9660
9665
9670
9675
9680
9685
9690
9695
9700
9705
9710
9715
9720
9725
9730
9735
9740
9745
9750
9755
9760
9765
9770
9775
9780
9785
9790
9795
9800
9805
9810
9815
9820
9825
9830
9835
9840
9845
9850
9855
9860
9865
9870
9875
9880
9885
9890
9895
9900
9905
9910
9915
9920
9925
9930
9935
9940
9945
9950
9955
9960
9965
9970
9975
9980
9985
9990
9995
10000
10005
10010
10015
10020
10025
10030
10035
10040
10045
10050
10055
10060
10065
10070
10075
10080
10085
10090
10095
10100
10105
10110
10115
10120
10125
10130
10135
10140
10145
10150
10155
10160
10165
10170
101

Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*, Vols. I, II and III, Second Edition (1989); Perbal, B., *A Practical Guide to Molecular Cloning* (1984); the series, *Methods In Enzymology* (S. Colowick and N. Kaplan eds., Academic Press, Inc.); and *Handbook of Experimental Immunology*, Vols. I-IV (D.M. Weir and C.C. Blackwell eds., 1986, Blackwell Scientific Publications).

All publications, patents and patent applications cited herein, whether *supra* or *infra*, are hereby incorporated by reference in their entirety.

A. Definitions

In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

It must be noted that, as used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to "an EHEC bacterium " includes a mixture of two or more such bacteria, and the like.

As used herein, the term EHEC "cell culture supernatant" or "CCS" refers to a supernatant derived from a cell culture of one or more EHEC serotypes, which supernatant is substantially free of EHEC bacterial cells or the lysate of such cells, and which contains a mixture of EHEC antigens that have been secreted into the growth media. Generally, an EHEC "CCS" will contain at least the secreted antigens EspA, EspB, EspD and Tir, and fragments or aggregates thereof. The CCS of the present invention may also include other secreted proteins, such as EspF and MAP, one or both of Shiga toxins 1 and 2, as well as EspP which is an approximately 100 kDa protein which is not secreted by the type III system. The proteins can be present in a native form, or a denatured or degraded form, so long as the CCS still functions to stimulate an immune response in the host subject such that EHEC disease is lessened or prevented, and/or colonization of EHEC is lessened or suppressed. In some instances, a CCS may be supplemented with additional recombinant or purified secreted antigens, such as with additional EspA, EspB, EspD and/or Tir, as well as with any of the other secreted proteins, and may also be supplemented with Intimin. In certain

embodiments, EspA + Tir will comprise at least 20% of the cell culture supernatant protein.

As used herein, a "recombinant" EHEC secreted protein, such as rEspA, rEspB, rEspD and rTir, as well as the "recombinant Intimin", refers to the full-length polypeptide sequence, fragments of the reference sequence or substitutions, deletions and/or additions to the reference sequence, so long as the proteins retain at least one specific epitope or activity. Generally, analogs of the reference sequence will display at least about 50% sequence identity, preferably at least about 75% to 85% sequence identity, and even more preferably about 90% to 95% or more sequence identity, to the full-length reference sequence. See, e.g., GenBank Accession Nos. AE005594, AE005595, AP002566, AE005174, NC_002695, NC_002655 for the complete sequence of the *E. coli* O157:H7 genome, which includes the sequences of the various O157:H7 secreted proteins. See, e.g., International Publication No. WO 97/40063, as well as GenBank Accession Nos. Y13068, U80908, U5681, Z54352, AJ225021, AJ225020, AJ225019, AJ225018, AJ225017, AJ225016, AJ225015, AF022236 and AF200363 for the nucleotide and amino acid sequences of EspA from a number of *E. coli* serotypes. See, e.g., International Publication No. WO 99/24576, as well as GenBank Accession Nos. AF125993, AF132728, AF045568, AF022236, AF70067, AF070068, AF013122, AF200363, AF113597, AF070069, AB036053, AB026719, U5904 and U59502, for the nucleotide and amino acid sequences of Tir from a number of *E. coli* serotypes. See, e.g., GenBank Accession Nos. U32312, U38618, U59503, U66102, AF081183, AF081182, AF130315, AF339751, AJ308551, AF301015, AF329681, AF319597, AJ275089-AJ275113 for the nucleotide and amino acid sequences of Intimin from a number of *E. coli* serotypes. See, e.g., GenBank Accession Nos. U80796, U65681, Y13068, Y13859, X96953, X99670, X96953, Z21555, AF254454, AF254455, AF254456, AF254457, AF054421, AF059713, AF144008, AF144009 for the nucleotide and amino acid sequences of EspB from a number of *E. coli* serotypes. See, e.g., GenBank Accession Nos. Y13068, Y13859, Y17875, Y17874, Y09228, U65681, AF054421 and AF064683, for the nucleotide and amino acid sequences of EspD from a number of *E. coli* serotypes.

"Homology" refers to the percent similarity between two polynucleotide or two polypeptide moieties. Two DNA, or two polypeptide sequences are "substantially homologous" to each other

when the sequences exhibit at least about 80%-85%, preferably at least about 90%, and most preferably at least about 95%-98% sequence similarity over a defined length of the molecules. As used herein, substantially homologous also refers to sequences showing complete identity to the specified DNA or polypeptide sequence.

5 Percent sequence identity can be determined by a direct comparison of the sequence information between two molecules by aligning the sequences, counting the exact number of matches between the two aligned sequences, dividing by the length of the shorter sequence, and multiplying the result by 100. Readily available computer programs can be used to aid in the analysis, such as ALIGN, Dayhoff, M.O. in *Atlas of Protein Sequence and Structure* M.O. Dayhoff ed., 5 Suppl. 3:353-358, National biomedical Research Foundation, Washington, DC, which adapts the local homology algorithm of Smith and Waterman (1981) *Advances in Appl. Math.* 2:482-489 for peptide analysis. Programs for determining nucleotide sequence identity are available in the Wisconsin Sequence Analysis Package, Version 8 (available from Genetics Computer Group, Madison, WI) for example, the BESTFIT, FASTA and GAP programs, which also rely on the Smith and Waterman algorithm. These programs are readily utilized with the default parameters recommended by the manufacturer and described in the Wisconsin Sequence Analysis Package referred to above. For example, percent identity of a particular nucleotide sequence to a reference sequence can be determined using the homology algorithm of Smith and Waterman with a default scoring table and a gap penalty of six nucleotide positions.

20 Alternatively, homology can be determined by hybridization of polynucleotides under conditions which form stable duplexes between homologous regions, followed by digestion with single-stranded-specific nuclease(s), and size determination of the digested fragments. DNA sequences that are substantially homologous can be identified in a Southern hybridization experiment under, for example, stringent conditions, as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Sambrook et al., *supra*; *DNA Cloning, supra*; *Nucleic Acid Hybridization, supra*.

25 As used herein, "vaccine" refers to a CCS composition that serves to stimulate an immune response to an EHEC antigen, such as a type III secreted EHEC antigen, therein. The immune

response need not provide complete protection and/or treatment against EHEC infection or against colonization and shedding of EHEC. Even partial protection against colonization and shedding of EHEC bacteria will find use herein as shedding and contaminated meat production will still be reduced. In some cases, a vaccine will include an immunological adjuvant in order to enhance the immune response. The term "adjuvant" refers to an agent which acts in a nonspecific manner to increase an immune response to a particular antigen or combination of antigens, thus reducing the quantity of antigen necessary in any given vaccine, and/or the frequency of injection necessary in order to generate an adequate immune response to the antigen of interest. See, e.g., A.C. Allison *J. Reticuloendothel. Soc.* (1979) 26:619-630. Such adjuvants are described further below.

As used herein, "colonization" refers to the presence of EHEC in the intestinal tract of a mammal, such as a ruminant.

As used herein, "shedding" refers to the presence of EHEC in feces.

As used herein, "therapeutic amount", "effective amount" and "amount effective to" refer to an amount of vaccine effective to elicit an immune response against a secreted antigen present in the CCS, thereby reducing or preventing EHEC disease, and/or EHEC colonization of a mammal such as a ruminant; and/or reducing the number of animals shedding EHEC; and/or reducing the number of EHEC shed by an animal; and/or, reducing the time period of EHEC shedding by an animal.

As used herein, "immunization" or "immunize" refers to administration of CCS, with or without additional recombinant or purified EHEC antigens such as EspA, Tir, EspB, EspD, and/or Intimin, in an amount effective to stimulate the immune system of the animal to which the CCS is administered, to elicit an immunological response against one or more of the secreted antigens present in the CCS.

The term "epitope" refers to the site on an antigen or hapten to which specific B cells and/or T cells respond. The term is also used interchangeably with "antigenic determinant" or "antigenic determinant site."

An "immunological response" to a composition or vaccine is the development in the host of a cellular and/or antibody-mediated immune response to the composition or vaccine of interest.

Usually, an “immunological response” includes but is not limited to one or more of the following effects: the production of antibodies, B cells, helper T cells, suppressor T cells, and/or cytotoxic T cells and/or $\gamma\delta$ T cells, directed specifically to an antigen or antigens included in the composition or vaccine of interest. Preferably, the host will display either a therapeutic or protective

5 immunological response such that EHEC disease is lessened and/or prevented; resistance of the intestine to colonization with EHEC is imparted; the number of animals shedding EHEC is reduced; the number of EHEC shed by an animal is reduced; and/or the time period of EHEC shedding by an animal is reduced.

10 The terms “immunogenic” protein or polypeptide refer to an amino acid sequence which elicits an immunological response as described above. An “immunogenic” protein or polypeptide, as used herein, includes the full-length sequence of the particular EHEC protein in question, analogs thereof, aggregates, or immunogenic fragments thereof. By “immunogenic fragment” is meant a fragment of a secreted EHEC protein which includes one or more epitopes and thus elicits the immunological response described above. Such fragments can be identified using any number
15 of epitope mapping techniques, well known in the art. See, e.g., *Epitope Mapping Protocols* in *Methods in Molecular Biology*, Vol. 66 (Glenn E. Morris, Ed., 1996) Humana Press, Totowa, New Jersey. For example, linear epitopes may be determined by e.g., concurrently synthesizing large numbers of peptides on solid supports, the peptides corresponding to portions of the protein molecule, and reacting the peptides with antibodies while the peptides are still attached to the
20 supports. Such techniques are known in the art and described in, e.g., U.S. Patent No. 4,708,871; Geysen et al. (1984) *Proc. Natl. Acad. Sci. USA* 81:3998-4002; Geysen et al. (1986) *Molec. Immunol.* 23:709-715, all incorporated herein by reference in their entireties. Similarly, conformational epitopes are readily identified by determining spatial conformation of amino acids such as by, e.g., x-ray crystallography and 2-dimensional nuclear magnetic resonance. See, e.g.,
25 *Epitope Mapping Protocols, supra*. Antigenic regions of proteins can also be identified using standard antigenicity and hydropathy plots, such as those calculated using, e.g., the Omega version 1.0 software program available from the Oxford Molecular Group. This computer program employs the Hopp/Woods method, Hopp et al., *Proc. Natl. Acad. Sci USA* (1981) 78:3824-3828 for

determining antigenicity profiles, and the Kyte-Doolittle technique, Kyte et al., *J. Mol. Biol.* (1982) 157:105-132 for hydropathy plots.

Immunogenic fragments, for purposes of the present invention, will usually include at least about 3 amino acids, preferably at least about 5 amino acids, more preferably at least about 10-15 amino acids, and most preferably 25 or more amino acids, of the parent EHEC secreted protein molecule. There is no critical upper limit to the length of the fragment, which may comprise nearly the full-length of the protein sequence, or even a fusion protein comprising two or more epitopes of the particular EHEC secreted protein.

“Native” proteins or polypeptides refer to proteins or polypeptides isolated from the source in which the proteins naturally occur. “Recombinant” polypeptides refer to polypeptides produced by recombinant DNA techniques; i.e., produced from cells transformed by an exogenous DNA construct encoding the desired polypeptide. “Synthetic” polypeptides are those prepared by chemical synthesis.

The term “treatment” as used herein refers to either (i) the prevention of infection or reinfection (prophylaxis), or (ii) the reduction or elimination of symptoms of the disease of interest (therapy).

By “mammalian subject” is meant any member of the class Mammalia, including humans and all other mammary gland possessing animals (both male and female), such as ruminants, including, but not limited to, bovine, porcine and *Ovis* (sheep and goats) species. The term does not denote a particular age. Thus, adults, newborns, and fetuses are intended to be covered.

B. General Methods

Central to the present invention is the discovery that cell culture supernatants derived from EHEC cultures which contain EHEC secreted antigens, produce an immune response in animals to which they are administered and thereby provide protection against EHEC infection, such as protection against colonization. In certain embodiments, the compositions comprise a mixture of EHEC secreted antigens, including but not limited to EspA, EspB, EspD and/or Tir. The CCS of the present invention may also include other secreted proteins, such as EspF and MAP, one or both

of Shiga toxins 1 and 2, as well as EspP which is an approximately 100 kDa protein which is not secreted by the type III system. In other embodiments, the CCS is supplemented with additional recombinant or purified EHEC antigens, such as with additional EspA, EspB, EspD, Tir, Intimin, and the like. In certain embodiments, EspA + Tir comprise at least 20% of the cell culture supernatant protein. The compositions can comprise cell culture supernatants and additional adjuvants from more than one EHEC serotype to provide protection against multiple EHEC organisms. Moreover, a pharmaceutically acceptable adjuvant may be administered with the cell culture supernatant. The compositions are administered in an amount effective to elicit an immune response to one or more of the secreted antigens, thereby reducing or eliminating EHEC infection. In some instances, EHEC colonization of the animal is reduced or eliminated. In preferred embodiments, the animal is a cow or a sheep or other ruminant. In particularly preferred embodiments, the cell culture supernatant is derived from a cell culture of EHEC O157:H7 or EHEC O157:NM.

Immunization with CCS stimulates the immune system of the immunized animal to produce antibodies against one or more secreted EHEC antigens, such as EspA, EspB, EspD and Tir, that block EHEC attachment to intestinal epithelial cells, interfere with EHEC colonization and, thereby, reduce EHEC shedding by the animal. This reduction in EHEC shedding results in a reduction in EHEC contamination of food and water and a reduction in EHEC-caused disease in humans. Moreover, the unexpected and surprising ability of CCS immunization to prevent, reduce and eliminate EHEC colonization and shedding by cattle addresses a long-felt unfulfilled need in the medical arts, and provides an important benefit for humans.

Additionally, the CCS of the present invention can be used to treat or prevent EHEC infections in other mammals such as humans. If used in humans, the CCS can be produced from a mutated EHEC which has been engineered to knock out one or both of the Shiga toxins 1 and 2 in order to reduce toxicity.

As explained above, the therapeutic effectiveness of CCS can be increased by adding thereto one or more of the secreted antigens in recombinant or purified form, such as by adding recombinant or purified EspA, EspB, EspD, Tir, and the like, fragments thereof and/or analogs

thereof. Intimin may also be added. Other methods to increase the therapeutic effectiveness of CCS include, but are not limited to, complexing the CCS to natural or synthetic carriers and administering the CCS, before, at the same time as, or after another anti-EHEC agent. Such agents include, but are not limited to, biological, biologically engineered, chemical, nucleic acid based and recombinant protein anti-EHEC agents.

CCS from pathogenic bacteria, other than serotypes of EHEC, that require proteins such as EspA and Tir to colonize a host, can also be used to stimulate the immune system of an animal to produce antibodies against secreted EHEC antigens that reduce bacterial binding to intestinal epithelial cells of the animal. These bacterial species include, but are not limited to *Citrobacter rodentium*.

The CCS for use herein may be obtained from cultures of any EHEC serotype, including, without limitation, EHEC serotypes from serogroups O157, O158, O5, O8, O18, O26, O45, O48, O52, O55, O75, O76, O78, O84, O91, O103, O104, O111, O113, O114, O116, O118, O119, O121, O125, O28, O145, O146, O163, O165. Such EHEC serotypes are readily obtained from sera of infected animals. Methods for isolated EHEC are well known in the art. See, e.g., Elder et al., *Proc. Natl. Acad. Sci. USA* (2000) 97:2999; Van Donkersgoed et al., *Can. Vet. J.* (1999) 40:332; Van Donkersgoed et al., *Can. Vet. J.* (2001) 42:714. Generally, such methods entail direct plating on sorbitol MacConkey agar supplemented with cefixime and tellurite or immunomagnetic enrichment followed by plating on the same media. Moreover, CCS may be obtained from EHEC serotypes that have been genetically engineered to knock-out expression of Shiga toxins 1 and/or 2, in order to reduce toxicity.

Generally, CCS is produced by culturing EHEC bacteria in a suitable medium, under conditions that favor type III antigen secretion. Suitable media and conditions for culturing EHEC bacteria are known in the art and described in e.g., U.S. Patent Nos. 6,136,554 and 6,165,743 (incorporated herein by reference in their entireties), as well as in Li et al., *Infect. Immun.* (2000) 68:5090-5095; Fey et al., *Emerg. Infect. Dis.* (2000) Volume 6. A particularly preferable method of obtaining CCS is by first growing organisms in Luria-Bertani (LB) medium for a period of about 8 to 48 hours, preferably about 12 to 24 hours, and diluting this culture about 1:5 to 1:50, preferably

1:5 to 1:25, more preferably about 1:10, into M-9 minimal medium supplemented with 20-100 mM NaHCO₂, preferably 30-50 mM, most preferably about 44 mM NaHCO₂, 4-20 mM MgSO₄, preferably 5-10 mM and most preferably about 8 mM MgSO₄, 0.1 to 1.5% glucose, preferable 0.2 to 1%, most preferably 0.4% glucose and 0.05 to 0.5% Casamino Acids, preferably 0.07 to 0.2%,
5 most preferably about 0.1% Casamino Acids. Cultures are generally maintained at about 37 degrees C in 2-10% CO₂, preferably about 5% CO₂, to an optical density of about 600nm of 0.7 to 0.8. Whole cells are then removed by centrifugation and the supernatant can be concentrated, e.g., 10-1000 fold or more, such as 100-fold, using dialysis, ultrafiltration and the like. Total protein is easily determined using methods well known in the art.

10 As explained above, the CCS can be supplemented with additional EHEC secreted proteins, such as EspA, EspB, EspD and/or Tir. Intimin may also be added. These proteins can be produced recombinantly using techniques well known in the art. See, e.g., International Publication Nos. WO 97/40063 and WO 99/24576 for a description of the production of representative recombinant EHEC secreted proteins. In particular, the sequences for EspA, EspB, EspD, Tir and Intimin from
15 various serotypes are known and described. See, e.g., GenBank Accession Nos. AE005594, AE005595, AP002566, AE005174, NC_002695, NC_002655 for the complete sequence of the *E. coli* O157:H7 genome, which includes the sequences of the various O157:H7 secreted proteins. See, e.g., International Publication No. WO 97/40063, as well as GenBank Accession Nos. Y13068, U80908, U5681, Z54352, AJ225021, AJ225020, AJ225019, AJ225018, AJ225017,
20 AJ225016, AJ225015, AF022236 and AF200363 for the nucleotide and amino acid sequences of EspA from a number of *E. coli* serotypes. See, e.g., International Publication No. WO 99/24576, as well as GenBank Accession Nos. AF125993, AF132728, AF045568, AF022236, AF70067, AF070068, AF013122, AF200363, AF113597, AF070069, AB036053, AB026719, U5904 and U59502, for the nucleotide and amino acid sequences of Tir from a number of *E. coli* serotypes.
25 See, e.g., GenBank Accession Nos. U32312, U38618, U59503, U66102, AF081183, AF081182, AF130315, AF339751, AJ308551, AF301015, AF329681, AF319597, AJ275089-AJ275113 for the nucleotide and amino acid sequences of Intimin from a number of *E. coli* serotypes. See, e.g., GenBank Accession Nos. U80796, U65681, Y13068, Y13859, X96953, X99670, X96953, Z21555,

AF254454, AF254455, AF254456, AF254457, AF054421, AF059713, AF144008, AF144009 for the nucleotide and amino acid sequences of EspB from a number of *E. coli* serotypes. See, e.g., GenBank Accession Nos. Y13068, Y13859, Y17875, Y17874, Y09228, U65681, AF054421 and AF064683, for the nucleotide and amino acid sequences of EspD from a number of *E. coli* serotypes.

These sequences can be used to design oligonucleotide probes and used to screen genomic or cDNA libraries for genes from other *E. coli* serotypes. The basic strategies for preparing oligonucleotide probes and DNA libraries, as well as their screening by nucleic acid hybridization, are well known to those of ordinary skill in the art. See, e.g., *DNA Cloning*: Vol. I, *supra*; *Nucleic Acid Hybridization*, *supra*; *Oligonucleotide Synthesis*, *supra*; Sambrook et al., *supra*. Once a clone from the screened library has been identified by positive hybridization, it can be confirmed by restriction enzyme analysis and DNA sequencing that the particular library insert contains a type III gene or a homolog thereof. The genes can then be further isolated using standard techniques and, if desired, PCR approaches or restriction enzymes employed to delete portions of the full-length sequence.

Similarly, genes can be isolated directly from bacteria using known techniques, such as phenol extraction and the sequence further manipulated to produce any desired alterations. See, e.g., Sambrook et al., *supra*, for a description of techniques used to obtain and isolate DNA. Alternatively, DNA sequences encoding the proteins of interest can be prepared synthetically rather than cloned. The DNA sequences can be designed with the appropriate codons for the particular amino acid sequence. In general, one will select preferred codons for the intended host if the sequence will be used for expression. The complete sequence is assembled from overlapping oligonucleotides prepared by standard methods and assembled into a complete coding sequence. See, e.g., Edge (1981) *Nature* 292:756; Nambair et al. (1984) *Science* 223:1299; Jay et al. (1984) *J. Biol. Chem.* 259:6311.

Once coding sequences for the desired proteins have been prepared or isolated, they can be cloned into any suitable vector or replicon. Numerous cloning vectors are known to those of skill in the art, and the selection of an appropriate cloning vector is a matter of choice. Examples of re-

combinant DNA vectors for cloning and host cells which they can transform include the bacteriophage λ (*E. coli*), pBR322 (*E. coli*), pACYC177 (*E. coli*), pKT230 (gram-negative bacteria), pGV1106 (gram-negative bacteria), pLAFR1 (gram-negative bacteria), pME290 (non-*E. coli* gram-negative bacteria), pHV14 (*E. coli* and *Bacillus subtilis*), pBD9 (*Bacillus*), pIJ61 (*Streptomyces*), pUC6 (*Streptomyces*), YIp5 (*Saccharomyces*), YCp19 (*Saccharomyces*) and bovine papilloma virus (mammalian cells). See, Sambrook et al., *supra*; *DNA Cloning, supra*; B. Perbal, *supra*.

The gene can be placed under the control of a promoter, ribosome binding site (for bacterial expression) and, optionally, an operator (collectively referred to herein as "control" elements), so that the DNA sequence encoding the desired protein is transcribed into RNA in the host cell transformed by a vector containing this expression construction. The coding sequence may or may not contain a signal peptide or leader sequence. Leader sequences can be removed by the host in post-translational processing. See, e.g., U.S. Patent Nos. 4,431,739; 4,425,437; 4,338,397.

Other regulatory sequences may also be desirable which allow for regulation of expression of the protein sequences relative to the growth of the host cell. Regulatory sequences are known to those of skill in the art, and examples include those which cause the expression of a gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Other types of regulatory elements may also be present in the vector, for example, enhancer sequences.

The control sequences and other regulatory sequences may be ligated to the coding sequence prior to insertion into a vector, such as the cloning vectors described above. Alternatively, the coding sequence can be cloned directly into an expression vector which already contains the control sequences and an appropriate restriction site.

In some cases it may be necessary to modify the coding sequence so that it may be attached to the control sequences with the appropriate orientation; i.e., to maintain the proper reading frame. It may also be desirable to produce mutants or analogs of the protein. Mutants or analogs may be prepared by the deletion of a portion of the sequence encoding the protein, by insertion of a sequence, and/or by substitution of one or more nucleotides within the sequence. Techniques for

modifying nucleotide sequences, such as site-directed mutagenesis, are described in, e.g., Sambrook et al., *supra*; *DNA Cloning, supra*; *Nucleic Acid Hybridization, supra*.

The expression vector is then used to transform an appropriate host cell. A number of mammalian cell lines are known in the art and include immortalized cell lines available from the American Type Culture Collection (ATCC), such as, but not limited to, Chinese hamster ovary (CHO) cells, HeLa cells, baby hamster kidney (BHK) cells, monkey kidney cells (COS), human hepatocellular carcinoma cells (e.g., Hep G2), Madin-Darby bovine kidney ("MDBK") cells, as well as others. Similarly, bacterial hosts such as *E. coli*, *Bacillus subtilis*, and *Streptococcus spp.*, will find use with the present expression constructs. Yeast hosts useful in the present invention include *inter alia*, *Saccharomyces cerevisiae*, *Candida albicans*, *Candida maltosa*, *Hansenula polymorpha*, *Kluyveromyces fragilis*, *Kluyveromyces lactis*, *Pichia guillerimondii*, *Pichia pastoris*, *Schizosaccharomyces pombe* and *Yarrowia lipolytica*. Insect cells for use with baculovirus expression vectors include, *inter alia*, *Aedes aegypti*, *Autographa californica*, *Bombyx mori*, *Drosophila melanogaster*, *Spodoptera frugiperda*, and *Trichoplusia ni*.

Depending on the expression system and host selected, the proteins of the present invention are produced by culturing host cells transformed by an expression vector described above under conditions whereby the protein of interest is expressed. The protein is then isolated from the host cells and purified. The selection of the appropriate growth conditions and recovery methods are within the skill of the art.

The proteins of the present invention may also be produced by chemical synthesis such as solid phase peptide synthesis, using known amino acid sequences or amino acid sequences derived from the DNA sequence of the genes of interest. Such methods are known to those skilled in the art. See, e.g., J. M. Stewart and J. D. Young, *Solid Phase Peptide Synthesis*, 2nd Ed., Pierce Chemical Co., Rockford, IL (1984) and G. Barany and R. B. Merrifield, *The Peptides: Analysis, Synthesis, Biology*, editors E. Gross and J. Meienhofer, Vol. 2, Academic Press, New York, (1980), pp. 3-254, for solid phase peptide synthesis techniques; and M. Bodansky, *Principles of Peptide Synthesis*, Springer-Verlag, Berlin (1984) and E. Gross and J. Meienhofer, Eds., *The Peptides: Analysis, Synthesis, Biology, supra*, Vol. 1, for classical solution synthesis. Chemical synthesis of

peptides may be preferable if a small fragment of the antigen in question is capable of raising an immunological response in the subject of interest.

Once the above cell culture supernatants and, if desired, additional recombinant and/or purified proteins are produced, they are formulated into compositions for delivery to a mammalian subject. The CCS is administered alone, or mixed with a pharmaceutically acceptable vehicle or excipient. Suitable vehicles are, for example, water, saline, dextrose, glycerol, ethanol, or the like, and combinations thereof. In addition, the vehicle may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, or adjuvants in the case of vaccine compositions, which enhance the effectiveness of the vaccine. Suitable adjuvants are described further below. The compositions of the present invention can also include ancillary substances, such as pharmacological agents, cytokines, or other biological response modifiers.

As explained above, vaccine compositions of the present invention may include adjuvants to further increase the immunogenicity of one or more of the EHEC antigens. Such adjuvants include any compound or compounds that act to increase an immune response to an EHEC antigen or combination of antigens, thus reducing the quantity of antigen necessary in the vaccine, and/or the frequency of injection necessary in order to generate an adequate immune response. Adjuvants may include for example, emulsifiers, muramyl dipeptides, avridine, aqueous adjuvants such as aluminum hydroxide, chitosan-based adjuvants, and any of the various saponins, oils, and other substances known in the art, such as Amphigen, LPS, bacterial cell wall extracts, bacterial DNA, synthetic oligonucleotides and combinations thereof (Schijns et al., *Curr. Opi. Immunol.* (2000) 12:456), *Mycobacterial phlei* (*M. phlei*) cell wall extract (MCWE) (U.S. Patent No. 4,744,984), *M. phlei* DNA (M-DNA), M-DNA-*M. phlei* cell wall complex (MCC). For example, compounds which may serve as emulsifiers herein include natural and synthetic emulsifying agents, as well as anionic, cationic and nonionic compounds. Among the synthetic compounds, anionic emulsifying agents include, for example, the potassium, sodium and ammonium salts of lauric and oleic acid, the calcium, magnesium and aluminum salts of fatty acids (i.e., metallic soaps), and organic sulfonates such as sodium lauryl sulfate. Synthetic cationic agents include, for example, cetyltrimethylammonium bromide, while synthetic nonionic agents are exemplified by glyceryl

esters (e.g., glyceryl monostearate), polyoxyethylene glycol esters and ethers, and the sorbitan fatty acid esters (e.g., sorbitan monopalmitate) and their polyoxyethylene derivatives (e.g., polyoxyethylene sorbitan monopalmitate). Natural emulsifying agents include acacia, gelatin, lecithin and cholesterol.

5 Other suitable adjuvants can be formed with an oil component, such as a single oil, a mixture of oils, a water-in-oil emulsion, or an oil-in-water emulsion. The oil may be a mineral oil, a vegetable oil, or an animal oil. Mineral oil, or oil-in-water emulsions in which the oil component is mineral oil are preferred. In this regard, a "mineral oil" is defined herein as a mixture of liquid hydrocarbons obtained from petrolatum via a distillation technique; the term is synonymous with
 10 "liquid paraffin," "liquid petrolatum" and "white mineral oil." The term is also intended to include "light mineral oil," i.e., an oil which is similarly obtained by distillation of petrolatum, but which has a slightly lower specific gravity than white mineral oil. See, e.g., *Remington's Pharmaceutical Sciences, supra*. A particularly preferred oil component is the oil-in-water emulsion sold under the trade name of EMULSIGEN PLUS™ (comprising a light mineral oil as well as 0.05% formalin,
 15 and 30 mcg/mL gentamicin as preservatives), available from MVP Laboratories, Ralston, Nebraska. Suitable animal oils include, for example, cod liver oil, halibut oil, menhaden oil, orange roughy oil and shark liver oil, all of which are available commercially. Suitable vegetable oils, include, without limitation, canola oil, almond oil, cottonseed oil, corn oil, olive oil, peanut oil, safflower oil, sesame oil, soybean oil, and the like.

20 Alternatively, a number of aliphatic nitrogenous bases can be used as adjuvants with the vaccine formulations. For example, known immunologic adjuvants include amines, quaternary ammonium compounds, guanidines, benzamidines and thiouroniums (Gall, D. (1966) *Immunology* 11:369-386). Specific compounds include dimethyldioctadecylammonium bromide (DDA) (available from Kodak) and N,N-dioctadecyl-N,N-bis(2-hydroxyethyl)propanediamine ("avridine").

25 The use of DDA as an immunologic adjuvant has been described; see, e.g., the Kodak Laboratory Chemicals Bulletin 56(1):1-5 (1986); *Adv. Drug Deliv. Rev.* 5(3):163-187 (1990); *J. Controlled Release* 7:123-132 (1988); *Clin. Exp. Immunol.* 78(2):256-262 (1989); *J. Immunol. Methods* 97(2):159-164 (1987); *Immunology* 58(2):245-250 (1986); and *Int. Arch. Allergy Appl. Immunol.*

68(3):201-208 (1982). Avidine is also a well-known adjuvant. See, e.g., U.S. Patent No. 4,310,550 to Wolff, III et al., which describes the use of N,N-higher alkyl-N',N'-bis(2-hydroxyethyl)propane diamines in general, and avidine in particular, as vaccine adjuvants. U.S. Patent No. 5,151,267 to Babiuk, and Babiuk et al. (1986) *Virology* 159:57-66, also relate to the use of avidine as a vaccine adjuvant.

Particularly preferred for use herein is an adjuvant known as "VSA3" which is a modified form of the EMULSIGEN PLUS™ adjuvant which includes DDA (see, U.S. Patent No. 5,951,988, incorporated herein by reference in its entirety).

CCS vaccine compositions can be prepared by uniformly and intimately bringing into association the CCS preparations and the adjuvant using techniques well known to those skilled in the art including, but not limited to, mixing, sonication and microfluidation. The adjuvant will preferably comprise about 10 to 50% (v/v) of the vaccine, more preferably about 20 to 40% (v/v) and most preferably about 20 to 30% or 35% (v/v), or any integer within these ranges.

The compositions of the present invention are normally prepared as injectables, either as liquid solutions or suspensions, or as solid forms which are suitable for solution or suspension in liquid vehicles prior to injection. The preparation may also be prepared in solid form, emulsified or the active ingredient encapsulated in liposome vehicles or other particulate carriers used for sustained delivery. For example, the vaccine may be in the form of an oil emulsion, water in oil emulsion, water-in-oil-in-water emulsion, site-specific emulsion, long-residence emulsion, sticky-emulsion, microemulsion, nanoemulsion, liposome, microparticle, microsphere, nanosphere, nanoparticle and various natural or synthetic polymers, such as nonresorbable impermeable polymers such as ethylenevinyl acetate copolymers and Hytrel® copolymers, swellable polymers such as hydrogels, or resorbable polymers such as collagen and certain polyacids or polyesters such as those used to make resorbable sutures, that allow for sustained release of the vaccine.

Furthermore, the polypeptides may be formulated into compositions in either neutral or salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the active polypeptides) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or organic acids such as acetic, oxalic, tartaric,

mandelic, and the like. Salts formed from free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

5 Actual methods of preparing such dosage forms are known, or will be apparent, to those skilled in the art. See, e.g., *Remington's Pharmaceutical Sciences*, Mack Publishing Company, Easton, Pennsylvania, 18th edition, 1990.

10 The composition is formulated to contain an effective amount of secreted EHEC antigen, the exact amount being readily determined by one skilled in the art, wherein the amount depends on the animal to be treated and the capacity of the animal's immune system to synthesize antibodies. The composition or formulation to be administered will contain a quantity of one or more secreted EHEC antigens adequate to achieve the desired state in the subject being treated. For purposes of the present invention, a therapeutically effective amount of a vaccine comprising CCS with or without added recombinant and/or purified secreted EHEC antigens, contains about 0.05 to 1500 μ g secreted EHEC protein, preferably about 10 to 1000 μ g secreted EHEC protein, more preferably about 30 to 500 μ g and most preferably about 40 to 300 μ g, or any integer between these values. EspA + Tir, as well as other EHEC antigens, may comprise about 10% to 50% of total CCS protein, such as about 15% to 40% and most preferably about 15% to 25%. If supplemented with rEspA + rTir, the vaccine may contain about 5 to 500 μ g of protein, more preferably about 10 to 250 μ g and most preferably about 20 to 125 μ g.

20 Routes of administration include, but are not limited to, oral, topical, subcutaneous, intramuscular, intravenous, subcutaneous, intradermal, transdermal and subdermal. Depending on the route of administration, the volume per dose is preferably about 0.001 to 10 ml, more preferably about 0.01 to 5 ml, and most preferably about 0.1 to 3 ml. Vaccine can be administered in a single dose treatment or in multiple dose treatments (boosts) on a schedule and over a time period appropriate to the age, weight and condition of the subject, the particular vaccine formulation used, and the route of administration.

25 Any suitable pharmaceutical delivery means may be employed to deliver the compositions

to the vertebrate subject. For example, conventional needle syringes, spring or compressed gas (air) injectors (U.S. Patent Nos. 1,605,763 to Smoot; 3,788,315 to Laurens; 3,853,125 to Clark et al.; 4,596,556 to Morrow et al.; and 5,062,830 to Dunlap), liquid jet injectors (U.S. Patent Nos. 2,754,818 to Scherer; 3,330,276 to Gordon; and 4,518,385 to Lindmayer et al.), and particle
5 injectors (U.S. Patent Nos. 5,149,655 to McCabe et al. and 5,204,253 to Sanford et al.) are all appropriate for delivery of the compositions.

If a jet injector is used, a single jet of the liquid vaccine composition is ejected under high pressure and velocity, e.g., 1200-1400 PSI, thereby creating an opening in the skin and penetrating to depths suitable for immunization.

10 The following examples will serve to further illustrate the present invention without, at the same time, however, constituting any limitation thereof. On the contrary, it is to be clearly understood that resort may be had to various other embodiments, modifications, and equivalents thereof which, after reading the description herein, may suggest themselves to those skilled in the art without departing from the spirit of the present invention and/or the scope of the appended
15 claims.

C. Experimental

Example 1

20 *Preparation of cell culture supernatant (CCS)*

Wild type EHEC O157:H7 were grown under conditions to maximize the synthesis of CCS proteins (Li et al., *Infect. Immun.* (2000) 68:5090). Briefly, an overnight standing culture of EHEC O157:H7 was grown in Luria-Bertani (LB) medium overnight at 37°C ($\pm 5\%$ CO₂). The culture was diluted 1:10 in M-9 minimal medium supplemented with 0.1% Casamino Acids, 0.4% glucose,
25 8 mM MgSO₄ and 44 mM NaHCO₂. Cultures were grown standing at 37°C in 5% CO₂ to an optical density at 600 nm of 0.7 to 0.8 (6-8 h). Bacteria were removed by centrifugation at 8000 rpm for 20 min at 4°C. The supernatant was concentrated 100 fold by ultrafiltration and total protein was determined by the bicinchoninic acid protein assay method.

Figure 1 shows molecular weight markers (lane 1) and a typical CCS protein profile obtained by electrophoresis of CCS in a SDS-10% polyacrylamide gel (SDS-PAGE) followed by Coomassie blue staining (lane 2). The positions of EspA (25 kD), EspB/EspD (40 kD), undegraded Tir (70 kD) and degraded Tir (55 kD) are indicated. As determined by densitometric analysis using an HP Scanjet 5100C and the ID software program from Advance American Biotechnology (Fullerton, CA, USA), EspA was about 5% undegraded Tir about 20% and degraded Tir about 6% of the total protein. However, the percentages of proteins determined by densitometric analysis of Coomassie blue stained SDS-polyacrylamide gels is not exact due to variations in background staining, variations in the uptake of the Coomassie blue stain, variations in the density of the bands, and other factors known to those skilled in the art.

Example 2

Preparation of recombinant proteins

The genes coding for EspA, EspB, Intimin and Tir were isolated (Li et al., *Infect. Immun.* (2000) 68:5090). A clinical isolate of EHEC O157:H7 was used as the source of DNA. EspA, EspB, Tir, and the region of *eae* encoding the 280 carboxyl-terminal amino acids of Intimin were amplified from chromosomal DNA using PCR to introduce unique restriction sites, followed by cloning into appropriate plasmids. The resulting plasmids were cleaved and ligated to create histidine-tagged fusions. Plasmids were electrocuted into an expression strain of *E. coli* and the *E. coli* were propagated (Ngeleka et al., *Infect. Immun.* (1996) 64:3118). Gene expression was driven using the Tac promoter following IPTG (isopropyl- β -D-thiogalactopyranoside) induction. Bacteria were pelleted, resuspended in Tris-buffered saline and lysed by sonication. The lysate was centrifuged to remove insoluble material and the histidine-tagged proteins were purified by passage through a solid-phase nickel affinity chromatography column that specifically binds proteins containing the histidine tag. All recombinant protein preparations were stored at -20°C until use.

The purity of the recombinant proteins was assessed by SDS-PAGE on 10% gels followed by Coomassie blue staining. Typical gel profiles of the chromatographically purified recombinant

(r) proteins are shown in Figure 2. rEspA (lane 2) rEspB (lane 3) and rIntimin (lane 4), were recovered in relatively pure form, but rTir (lane 5) was subject to some degradation.

Example 3

5 *Vaccine formulation and delivery*

Vaccines were formulated by mixing CCS or rEspA + rTir in 2 ml of a carrier containing from 30 to 40% of an adjuvant. Vaccines were delivered subcutaneously. Animals were immunized on day 1 and again at a 3-4 week intervals (boost). Serum samples were obtained prior to the first immunization, at the time of each boost and at the end of the experiment.

10 The serological response to immunization was determined using an enzyme-linked immunosorbent assay (ELISA). One hundred μ l of rEspA (0.16 μ g/well), rTir (0.1 μ g/well), rEspB (0.24 μ g/well) and rIntimin (0.187 μ g/well) were used to coat the wells in microtiter plates and the plates were incubated overnight at 4°C. The wells were washed 3X, blocked with 0.5% nonfat dried milk in phosphate-buffered saline. Serial dilutions of sera were added to each well and
15 incubated for 2 h at 37°C. The wells were washed and blocked and 100 μ l of peroxidase-conjugated rabbit anti-bovine immunoglobulin G antibodies (1:5000) were added to each well for 1 h at 37°C. The wells were washed and plates were read at a wavelength of 492 nm.

Example 4

20 *Experimental Animals*

Cattle, between the ages of 8 and 12 months, were purchased from local ranchers. Fecal samples were obtained daily from each animal for 14 days. The number of EHEC O157:H7 in the fecal samples was determined by plating on Rainbow Agar. The plates were incubated at 37°C for 2 days and black colonies were enumerated. Growth was scored from 0-5. Animals having a score
25 of 0 (no EHEC O157:H7) were used in all experiments.

Example 5

Animal Colonization Model

A model for EHEC O157:H7 colonization of cattle, wherein the infection was sustained for >2 months, was developed using a dose-titration protocol.

5 EHEC O157:H7 were grown as in Example 1. Twenty-four cattle were divided into 3 groups of 8 animals each. Group 1 received 10^6 , Group 2 10^8 and Group 3 10^{10} CFU of EHEC O157:H7 by oral-gastric intubation in a volume of 50 ml on day 0.

To monitor shedding, fecal material was collected on days 1 through 14. The fecal material was weighed, suspended in sterile saline and inoculated into culture media. Culture density was
10 determined as in Example 1.

As shown in Figure 3, there was no significant difference between numbers of EHEC O157:H7 shed by Group 2 (10^8 CFU) and Group 3 (10^{10} CFU) cattle. Group 2 cattle shed the most EHEC O157:H7 on each of the 14 days. The number of EHEC O157:H7 shed by Group 2 cattle reached a maximum on day 6 and declined to zero by day 14.

15 Animals shedding EHEC O157:H7 (hereinafter, "positive") were kept an additional 40 days during which time the number of EHEC O157:H7 shed decreased to an undetectable level. The shedding of EHEC O157:H7 by previously positive animals (hereinafter, "carriers") was reactivated by withholding feed for 24 hours and vaccinating with commercially-available clostridial or *H. somnus* vaccines. As shown in Figure 4, the number of carrier animals shedding EHEC O157:H7
20 reached a maximum of approximately 50% on days 6 and 7 post-reactivation and declined to zero by day 15.

As a dose of 10^8 CFU produced a detectable number of shed EHEC O157:H7 during the 14 days post-infection (Figure 3) and resulted in persistently infected animals (Figure 4), this dose was used as the challenge dose in subsequent experiments.

Example 6

Protective capacity of CCS

To test the vaccine potential of secreted proteins, CCS was mixed with the oil-based adjuvant, VSA3 (U.S. Patent No. 5,951,988, incorporated herein by reference in its entirety; S. van Drunen Littel-van den Hurk et al., *Vaccine* (1993) 11:25) such that each 2 ml dose contained 200 µg of CCS protein and 30% (v/v) of adjuvant (CCS vaccine). For the control group, sterile saline was mixed with VSA3, such that each 2 ml dose contained 0 µg of CCS protein and 30% (v/v) of adjuvant (saline vaccine).

Sixteen cattle were divided in 2 groups of eight animals each. Group 1 cattle received 2 ml of CCS vaccine subcutaneously (experimental) and Group 2 cattle received 2 ml saline vaccine subcutaneously (control) on days 1 and 22 (boost). Seroconversion was assayed by ELISA (Example 3), on days 1 (pre-immunization), 22 and 36. As shown in Table 1, at day 22, Group 1 animals showed specific antibody titers to EspA and Tir and, at day 36, these titers showed a significant increase. Group 2 animals showed no specific antibody titers at days 22 and 36. In particular, the group which received the EHEC vaccine showed a 13-fold increase in specific antibody titer to type III secreted proteins after a single immunization and following the first booster, the eight animals in the EHEC vaccine group demonstrated a 45-fold increase in specific antibody titer while only one of the placebo vaccine group seroconverted (X^2 , $p=0.0002$).

Table 1
Serological response to immunization with CCS

		Specific Antibody Titers* - Group Means		
Group		Pre-immunization (Day 1)	Boost (Day 22)	Challenge (Day 36)
5	1. Experimental	350	5,000	12,500
	2. Control	450	500	650

*Values are group means expressed as the reciprocal of the highest dilution yielding a positive result.

At day 36, Group 1 and Group 2 animals were challenged with 10^8 CFU of EHEC O157:H7 by oral-gastric intubation and fecal shedding was monitored for 14 days (Example 5). As summarized in Table 2, fewer experimental animals shed EHEC O157:H7 than control animals and experimental animals that did shed, shed EHEC O157:H7 for a shorter period of time than control animals (Figure 7). In particular, The median number of days during which the organism was shed in the vaccinated animals was 1.5 compared to 3.5 in the placebo group (Wilcoxin Signed Rank Test, $p=0.08$). Seven out of eight placebo-immunized animals shed the bacteria during the trial and four of those animals shed the bacteria for four or more consecutive days, indicating that they were persistently infected. Five out of eight EHEC vaccine-immunized animals shed bacteria at some point during the trial but only one animal shed the organism for more than two consecutive days, indicating that colonization was transient and significantly less than the placebo group. The total number of bacteria isolated from fecal samples was significantly lower among the EHEC-vaccinated group as compared to the placebo group (Wilcoxin Signed Rank Test, $p=0.05$), with the former having a median of 6.25 colony forming units (CFU) per gram of feces recovered compared to a median value of 81.25 CFU/g for the latter. Thus, vaccination with the type III-secreted proteins appeared to reduce the ability of the organism to colonize the intestine as reflected by the decrease in the number of days animals shed the organism as well as the numbers of shed bacteria detected by fecal culture.

Table 2
Shedding by experimental and control animals

	Experimental	Control
Animals shedding >1 day	1/8	6/8
5 Number of days with scores of >1	1	8
Average days of shedding per animal	0.875	2.5
Total days shedding per group	7	20

These data show that CCS induced an antibody response in cattle that reduced both number of animals shedding EHEC O157:H7 and the number of days during which EHEC O157:H7 were shed.

In order to enhance the effectiveness of the vaccine formulation, groups of 6 calves were immunized as described above with one of three doses of secreted proteins (50 µg, 100 µg, 200 µg) or a placebo and the serological response was measured in serum samples taken at days 0, 21 (boost) and 35. No significant difference in anti-EHEC, anti-Tir or anti-EspA responses were observed between any of the groups which received the EHEC vaccine at any time point but all three were significantly higher than the placebo group on days 21 and 35. Thus, a second vaccine trial was designed in which three groups of yearling cattle were immunized three times with 50 µg of secreted proteins (n=13), 50 µg of secreted proteins from a *tir* mutant (Δ Tir, n=10) or a placebo (n=25). The adjuvant used was VSA3 and animals were immunized by subcutaneous injection on days 0, 21, and 35, followed by oral challenge with *E. coli* O157:H7 on day 49. The serological response to immunization is shown in Table 3 (days 0 and 49 only) and was comparable to that observed in the trial described above. The group which received the Δ Tir vaccine showed a response of similar magnitude against total secreted proteins as the group which received the vaccine prepared from the wild-type strain, but, as expected, a significantly reduced response to Tir (Wilcoxin Signed Rank Test, p=0.006). However, the former group did show an increase in anti-Tir antibody levels (Wilcoxin Signed Rank Test, p=0.009), indicating either exposure to an organism producing an immunologically related molecule or natural exposure to *E. coli* O157:H7.

This is further supported by the observation that there was a significant increase in the anti-Tir antibody titer in the placebo group on the day of challenge (Wilcoxin Signed Rank Test, $p=0.002$) but no difference between the placebo or Δ Tir groups ($p=0.37$, Kruskal-Wallis ANOVA). The response to EspA was similar in both the EHEC and Δ Tir vaccine groups ($p=0.45$, Kruskal-Wallis ANOVA) and was significantly higher than the placebo-immunized animals ($p<0.0001$).

Table 3. Median serological response to immunization with secreted proteins prepared from wild-type *E. coli* O157:H7 (EHEC), an isogenic *tir* mutant (Δ Tir) or a placebo. Titers are expressed as geometric mean values of the last positive dilution of sera (). Numbers in parentheses represent the 25th –75th percentile.

Group	n	Anti-EHEC		Anti-Tir		Anti-EspA	
		Day 0	Day 49	Day 0	Day 49	Day 0	Day 49
EHEC	13	10 (10-100)	6400 (3200-12800)	100 (10-200)	1600 (800-3200)	100 (10-200)	400 (200-1600)
Δ Tir	10	10 (10-100)	6400 (3200-25600)	10 (10-200)	200 (100-800)	100 (10-200)	300 (100-1600)
Placebo	25	10 (10-200)	10 (10-200)	100 (10-200)	200 (10-400)	100 (10-200)	100 (10-200)

The immune response against each vaccine formulation was also analyzed qualitatively by Western blotting using sera from two representative animals per group. The results for representative animals are shown in Figure 8 and demonstrate that the proteins secreted by the type III system were highly immunogenic in cattle. The response in the EHEC and Δ Tir vaccine groups was similar with the exception of the response against Tir which was absent in the latter group (Figure 8, top panels). EspB, EspD and Tir were all reactive, and following the second immunization on day 21 a significant response against lipopolysaccharide was also observed. The kinetics of the immune response in a vaccinated animal (Figure 8, bottom panels) show that anti-Tir antibodies were detectable following a single immunization, as were antibodies against 43-kDa and 100-kDa proteins. The latter proteins were produced by the wild-type strain as well as the *sepB* and *tir* mutants and the 100 kDa protein is probably EspP, a non-type III EHEC secreted protein.

Following oral challenge with *E. coli* O157:H7 on day 49, each group was monitored daily for fecal shedding of the organism for 14 days. In this experiment, bacteria were cultured following immunomagnetic enrichment (J. Van Donkersgoed et al., *Can. Vet. J.* (2001) 42:714; Chapman and Siddons, *J. Med. Microbiol.* (1996) 44:267) rather than direct plating since yearling cattle shed less than calves in this infection model. On the day of challenge, two animals in the placebo group were culture-positive for *E. coli* O157:H7 and were eliminated from the trial. The placebo-immunized animals shed the organism after challenge much more than those in the two EHEC vaccine groups (Figure 9). Those which received the placebo vaccine shed the organism for a median of 4 days, significantly longer than the median of 0 days by the other two vaccine groups ($p=0.0002$, Kruskal-Wallis ANOVA). Significantly fewer bacteria were recovered from the EHEC and Δ Tir vaccine groups ($p=0.04$, Kruskal-Wallis ANOVA). From day 2 post-infection onwards, 78% of the placebo animals shed the organism for at least one day as compared to 15% of the EHEC and 30% of the Δ Tir vaccinates (Table 4).

The data presented above demonstrate that virulence factors of EHEC, namely those secreted by the type III system, can be used as effective vaccine components for the reduction of colonization of cattle by EHEC bacteria, such as EHEC O157:H7. These proteins are major targets of the immune response in humans following infection (Li et al., *Infect. Immun.* (2000) 68:5090),

although cattle do not usually mount a significant serological response against these proteins following natural exposure to the organism. However, animals vaccinated with these proteins are primed and show an increase in anti-EHEC and anti-Tir titers following oral challenge with the organism.

5 Tir is likely required for colonization of the bovine intestine, and this is supported by the observation that a vaccine containing secreted proteins from a Δ Tir *E. coli* O157:H7 strain was not as efficacious as an identical formulation from an isogenic wild-type isolate. However, the former vaccine was significantly more efficacious than a placebo suggesting that immunity against colonization is multifactorial in nature. This is supported by the Western blot analysis of the
10 response to immunization in which several protein components as well as lipopolysaccharide were recognized. The contribution to protection by lipopolysaccharide is not known, but the presence of antibodies against this molecule does not correlate with protection in a murine EHEC model (Conlan et al., *Can. J. Microbiol.* (1999) 45:279; Conlan et al., *Can. J. Microbiol.* (2000) 46:283). Also, immunization with recombinant Tir and EspA can reduce numbers of bacteria shed, but not
15 the actual numbers of animals nor the duration of shedding.

The prevalence of non-O157 serotypes in North America appears to be increasing and represents a significant portion of EHEC infections in other geographical locations. Since the type III-secreted antigens appear to be relatively conserved among non-O157 EHEC serotypes, this vaccine formulation is likely broadly cross-protective, in contrast to formulations based upon the
20 O157 LPS antigen.

Table 4. Number of animals shedding *E. coli* O157:H7 at any time between day 2 and day 14 post-challenge.

Vaccine	Number Shedding	n	Percent Shedding	p-value
EHEC	2	13	15.4	0.003
ΔTir	3	10	30	0.008
Placebo	18	23	78.3	1

Example 7

Protective capacity of rEspA + rTir and rEspB + rIntimin

rEspA, rTir, rEspB and rIntimin were mixed with the oil-based adjuvant, VSA3, such that each 2 ml dose contained 50 μg of rEspA + rTir or of rEspB + rIntimin and 30% (v/v) of adjuvant. Sterile saline was mixed with VSA3, such that each 2 ml dose contained 0 μg of rEspA + rTir or of rEspB + rIntimin and 30% (v/v) of adjuvant.

Thirty four cattle were divided in 4 groups. Ten cattle, Group 1, were immunized with rEspA + rTir vaccine (experimental) and 10 cattle, Group 2, were immunized with rEspB + rIntimin vaccine (experimental) on days 1, 22 (boost) and 36. Seven cattle, Group 3, and 7 cattle, Group 4, were immunized with saline vaccine (control) on days 1, 22 (boost) and 36. Seroconversion was assayed by ELISA (Example 3) on days 1 (pre-immunization), 22 and 36. As shown in Figure 5, at day 22, Group 1 animals showed specific antibody titers to rEspA and to rTir and Group 2 animals showed specific antibody titers to rEspB and to rIntimin. Also, as shown in Figure 5, at day 36, Group 1 animals showed an increase in specific antibody titer to rTir and no change in specific antibody titer to rEspA and Group 2 animals showed an increase in specific antibody titer to rIntimin and a decrease in specific antibody titer to rEspB. Groups 3 and 4 animals showed no specific antibody titers at days 22 and 36.

At day 36, Groups 1-4 animals were challenged with 10^8 CFU of EHEC O157:H7 and shedding was monitored daily for 14 days (Example 5). As shown in Figure 6, differences in shedding between Group 1 (rTir+ rEspA) animals and Group 3 (saline) animals was minimal during the first 5 days post-challenge. However, during the second week post-challenge differences in Group 1 animals and Group 3 animals were evident. Fewer Group 1 animals shed EHEC O157:H7 than Group 3 animals. Group 1 animals shed less EHEC O157:H7 in their feces for shorter time periods than Group 3 animals. Differences in shedding between Group 2 (rEspB + rIntimin) and Group 4 (saline) animals were not evident with respect to the number of animals shedding, the number of EHEC O157:H7 shed and the time period of shedding.

These data show that the antibody response induced by rEspA + rTir vaccine interfered with EHEC O157:H7 colonization of cattle, whereas the antibody response induced by rEspB + rIntimin vaccine did not interfere with EHEC O157:H7 colonization of cattle.

Example 8

Protective capacity of CCS + rEspA + rTir

CCS, CCS + rEspA, CCS + rTir, CCS + rEspA + rTir and saline are mixed with an adjuvant.

Twenty-five cattle are divided into 5 groups of five 5 cattle and are immunized on days 1 and 22 (boost). Group 1 receives CCS vaccine, Group 2 CCS + rEspA vaccine, Group 3 CCS + rTir vaccine, Group 4 CCS + rEspA + rTir vaccine, and Group 5 saline vaccine. Seroconversion is assayed by ELISA (Example 3) on days 1 (pre-immunization), 22 (boost) and 36. On days 22 and 36 each of Groups 1-5 animals show specific antibody titers against EspA and Tir, whereas Group 6 animals show no specific antibody titers.

At day 36, Groups 1-5 animals are challenged with 10^8 CFU of EHEC O157:H7 and shedding is monitored daily for 14 days (Example 5). Fewer animals in Groups 1-4 shed EHEC O157:H7 than animals in Group 5. Group 5 animals shed the most EHEC O157:H7; Group 1 animals shed less EHEC O157:H7 than Group 5 animals and Groups 2-4 animals shed less EHEC O157:H7 than Group 1 animals.

Example 9

Protective capacity of CCS with various antigens

CCS is mixed with and adjuvant, such that each 2 ml dose contains 0, 50, 100 or 200 µg of
5 CCS and 30% (v/v) of adjuvant (Table 5).

Table 5

Protective capacity of CCS with various adjuvants

Antigen	Group	µg	Adjuvant
CCS	1	50	Emulsigen-Plus
CCS	2	100	Emulsigen-Plus
CCS	3	200	Emulsigen-Plus
CCS	4	200	Carbigen
CCS	5	100	MCC
CCS	6	200	MCC
CCS	7	200	MCC + Carbigen
CCS	8	200	VSA
CCS	9	0 (control)	Emulsigen-Plus

Seventy-two cattle are divided in 9 groups of 8 cattle. Groups 1-8 animals are immunized
20 with CCS + adjuvant (Table 5) and Group 9 cattle are immunized with saline + adjuvants on days 1
and 22 (boost). Seroconversion is assayed by ELISA (Example 3) on days 1 (pre-immunization),
22 (boost) and 36. Groups 1-8 (CCS + adjuvant) animals show specific antibody titers to EspA and
Tir on days 22 and 36. Group 9 (saline + adjuvant) animals show no specific antibody titers on
days 22 and 36.

Example 10

Protective capacity of CCS in dairy cows

Twenty adult dairy cows are divided in 2 groups of 10 cows. Group 1 is immunized with CCS vaccine and Group 2 is immunized with saline-vaccine on days 1 and day 22 (boost).

5 Seroconversion is assayed by ELISA (Example 3) on days 1 (pre-immunization), 22 and 36. On days 22 and 36 Group 1 cows show specific antibody titers against EspA and Tir, whereas Group 2 cows show no specific antibody titers.

At day 36, Groups 1 and 2 cows are challenged with 10^8 CFU of EHEC O157:H7 and shedding is monitored daily for 14 days (Example 5). Fewer Group 1 cows shed EHEC O157:H7 than Groups 2 cows. Group 1 cows shed less EHEC O157:H7 for a shorter period of time than Groups 2 cows.

Six months after the initial immunization, Group 1 and 2 cows are again immunized (2nd boost) via the subcutaneous route. On day 14 following the 2nd boost, antibody titers are assayed by ELISA (Example 3). Group 1 cows have specific antibody titers to EspA and Tir, whereas Group 2 cows have no specific antibody titers.

On day 14 following the 2nd boost, Groups 1 and 2 cows are again challenged with 10^8 CFU of EHEC O157:H7 and shedding is monitored daily for 14 days (Example 5). Fewer Group 1 (CCS) cows shed EHEC O157:H7 than Group 2 (saline) cows. Group 1 cows shed less EHEC O157:H7 for a shorter time periods than Group 2 cows.

Example 11

Protective capacity of CCS in calves

Ten weaned calves (3-6 month old) are divided into 2 groups of 5 calves and are immunized prior to entry into a feed-lot (day 0) and on the day of entry into a feed lot (day 1, boost). Group 1 calves receive CCS vaccine and Group 2 calves receive saline vaccine. Seroconversion is assayed by ELISA (Example 3) on days 0, 1 and 14. On days 1 and 14 Group 1 (CCS) calves show specific antibody titers to EspA and Tir, whereas Group 2 (saline) calves show no specific antibody titers.

At day 14, Groups 1 and 2 calves are challenged with 10^8 CFU of EHEC O157:H7 and shedding is assayed daily for 14 days (Example 5). Fewer Group 1 calves shed EHEC O157:H7 than Group 2 calves. Group 1 calves shed less EHEC O157:H7 for a shorter time period than Group 2 calves.

5 Ten weaned calves (3-6 month old) we divided into 2 groups of 5 calves and are immunized on the day of entry into a feed-lot (day 1) and on day 22 (boost) in the feed lot. Group 1 calves receive CCS vaccine and Group 2 calves receive saline vaccine. Seroconversion is assayed by ELISA (Example 3) on days 1 (pre-immunization), 22 and 36. On days 22 and 36 Group 1 (CCS) calves show specific antibody titers to EspA and Tir, whereas Group 2 (saline) calves show no specific antibody titers.

10 At day 36, Groups 1 and 2 calves are challenged with 10^8 CFU of EHEC O157:H7 and shedding is assayed daily for 14 days (Example 5). Fewer Group 1 calves shed EHEC O157:H7 than Group 2 calves. Group 1 calves shed less EHEC O157:H7 for a shorter time period than Group 2 calves.

Example 12

Protective capacity of CCS in sheep

20 Twenty adult sheep are divided in 2 groups of 10 sheep. Group 1 is immunized with CCS vaccine and Group 2 is immunized with saline vaccine on day 1 and day 22 (boost).

Seroconversion is assayed by ELISA (Example 3) on days 1 (pre-immunization), 22 and 36. On days 22 and 36 Group 1 sheep show specific antibody titers against EspA and Tir, whereas Group 2 sheep show no specific antibody titers.

25 At day 36, Groups 1 and 2 sheep are challenged with 10^8 CFU of EHEC O157:H7 and shedding is monitored daily for 14 days (Example 5). Fewer Group 1 sheep shed EHEC O157:H7 than Group 2 sheep. Group 1 sheep shed less EHEC O157:H7 for a shorter period of time than Group 2 sheep.

Thus, compositions and methods for treating and preventing enterohemorrhagic *E. coli* colonization of mammals have been disclosed. Although preferred embodiments of the subject invention have been described in some detail, it is understood that obvious variations can be made without departing from the spirit and the scope of the invention as defined by the appended claims.

5

20030750.010302